**EVALUATION OF DROUGHT TOLERANCE IN SELECTED UPLAND LAND RICE VARIETIES USING SIMPLE SEQUENCE (SSR) REPEAT MARKERS**

# ABSTRACT

Rice (*Oryza sativa* L.) is recognized as one of the most important staple food crops for more than 3.5 billion people in the world, accounting for more than half of human calories intake globally. The devastation caused by drought is one of the most important abiotic stress affecting rice production. The study was undertaken to identify drought tolerance upland rice varieties associated with polymorphic microsatellite (Simple Sequence Repeats (SSRs)) markers. The results on allele frequency, number of alleles and the polymorphic information content (PIC) of each locus among the studied varieties indicated that all the 6 specific primers produced scorable amplification bands in the analysis and all the primers were polymorphic, these polymorphic primers produced 23 alleles. The primer RM390 (0.73) showed higher discriminatory power to distinguish varieties due to its high PIC value and RM36 (0.19) showed lower PIC value. Indicating less discriminatory power of this primer under study. Gene diversity values of 20 rice varieties ranged from 0.19 (RM36) to 0.73 (RM390) with a mean of 0.58. These values showed that the varieties had several different recognized genes related to drought tolerance. The observed heterozygosity ranged from 0 to 0.33 (RM3558) with an average of 0.06 across all loci. The RM3558 and RM390 loci were the only ones that detected heterozygotes in the analyzed varieties. The majority of the SSR markers exhibited observed heterozygosity as zero, indicating that majority of rice cultivars used were pure and completely homozygous for SSR markers used in the study. UPGMA cluster analysis based on Jaccard’s dissimilarity coefficient showing 20 rice varieties generated from 6 SSR markers clustering model approach, distance-based neighbor-joining cluster and principal coordinate analysis using genotypic data grouped the varieties into three sub-populations. Analysis of molecular variance (AMOVA) and pairwise FST values showed significant differentiation among all the pairs of sub-population ranging from 0.152 to 0.222 suggesting that all the three subpopulations were significantly different from each other. Among the markers, RM3558, RM390 and RM170 were validated as markers linked to drought tolerance due to their ability to differentiate tolerant varieties from susceptible ones. Based on the SSRs variation analysis and clustering patterns, the NERICA rice varieties were identified as diverse cultivars and they are suitable and adapted to areas with insufficient rain fall for upland rice production and future breeding program.

**Keywords: Drought Stress, Drought tolerance, upland rice varieties and SSR markers**

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## 1.1 Introduction

Rice (*Oryza sativa* L.) is one of the most important staple food crops for more than 3.5 billion people in the world [12]. It is recognized as one of the major food crops, accounting for more than half of human calories intake globally. Rice is generally valued for its high nutritional benefits apart from being rich in calories, it is high in fiber, vitamins and minerals and low in cholesterol and sodium, suggesting it is a healthy source of energy [21].

Drought stress is one the most limiting factors for crop production in many regions of the world. It is a worldwide problem and has been shown to seriously influence grain production and quality of rice [28]. Particularly, drought conditions lead to a reduction in plant growth by affecting various physiological and biochemical processes [20]. Furthermore, most cultivated rice varieties are susceptible to drought, therefore, there is a need for continued improvement of rice cultivars to overcome this problem of low yield due to drought stress conditions [20]. The growth and development of rice is mainly sensitive to water-limited conditions due to the lower ability of taking up resources compared to other crops [9].

Improvement of molecular markers and their use for genetic analysis of agronomically important traits have been identified as a powerful tool for studying complex plant traits such as drought tolerance [19]. Principally, DNA-based molecular markers have been reliably used with availability of a huge number of polymorphic markers that enable precise classification of the varieties [24]. Improvement of rice for drought tolerance using conventional breeding methods is slow due to geographical differences and the variations of seasons in drought timing and severity, the intricate nature of drought tolerance traits and the difficulty in selection of combinations of traits [8]. Other aspects that have slowed down this process include the low heritability, multiple gene control as well as varieties and environmental interactions [4]. The use of molecular markers to select varieties possessing genes and genomic regions that control target traits can fast-track the progress in identifying drought tolerant rice. This is because molecular markers are consistently transmitted from generation to generation and are not subject to environmental influences [1] Studies have, however, utilized molecular markers to identify cultivars with traits directly related to drought tolerance [1]. These markers including Restriction Fragment Length Polymorphism (RFLP), Random Amplification of Polymorphic DNA (RAPD), Simple Sequence Repeats (SSRs), Inter Simple Sequence Repeats (ISSRs), Amplified Fragment Length Polymorphism (AFLP) and Single Nucleotide Polymorphisms (SNPs) have been available to assess the diversity at molecular level and have been used to enhance traditional breeding programs to improve rice crop [23]. Of all these, however, Simple sequence Repeats (SSRs) are considered the markers of choice in many areas of genetic diversity studies in rice due to their efficiency, abundance in rice genome, high level of polymorphism and high but simple reproducible assays that are reliable [22]. Therefore, the main aim of this study was to identify drought tolerance in selected upland rice varieties using Simple Sequence Repeat (SSR) Markers that can be used by rice breeders and farmers for future studies.

**1.2 Materials and Methods**

###  1.2.1 DNA Extraction

Twenty Upland rice varieties obtained from Liberia and Kenya were used in this experiment. Rice seedlings were harvested 14 days after germination for DNA extraction. The genomic DNA was isolated using Qiagen plant DNA extraction Kit (DNeasy Plant Mini Kit, Qiagen, Valencia, CA, USA) at the University of Eldoret, School of Agriculture, Biotechnology Laboratory.

###  1.2.2 DNA Quantification

The quality and quantity of the extracted DNA was determined using Nanodrop2000 (Thermos Fisher Scientific Inc., Carlsbad, CA, USA).

### 1.2.3 PCR Amplification Using Simple Sequence Repeat (SSR) Primers

Six (6) SSR specific primers previously reported to be linked to rice drought tolerance were used to screen the upland rice varieties [26]; [29] (Table 1).

**Table 1: List of Primers Linked to Drought Tolerance in Rice**

|  |
| --- |
| Primer Name Primer Sequence Chromosome no. Expected band size  |

RM38 F: ACGAGCTCTCGATCAGCCTA 8 250

 R: TCGGTCTCCATGTCCCAC

RM390 F: CTGGTTAACGTGAGAGCTCG 9 140

 R: GCAGATCAATTGGGGAGTAC

RM583 F: AGATCCATCCCTGTGGAGAG 1 192

 R: GCGAACTCGCGTTGTAATC

RM36 F: CAACTATGCACCATTGTCGC 3 192

 R: GTACTCCACAAGACCGTACC

RM170 F: TCGCGCTTCTTCCTCGTCGACG 6 121

 R: CCCGCTTGCAGAGGAAGCAG

RM3558 F: TTAGGTGTGTGAGCGTGGC 6 181

 R: ATACACAGATGACGCACACG

The PCR reaction constituted; Firepol Master Mix 4 *u*l, 1.0*u*l forward primer, 1.0 ul reverse primer, 2.5*u*l template DNA and 12.5*u*l DNase free water in a total volume of 20.0ul. The reaction was then carried out in Eppendorf mastercycler epgradient S PCR. The PCR program consisted of 94oc initial denaturation for 3 min, 94°c denaturation for 30 seconds, varied annealing temperature depending on the primer (between 49-60 °c) for 1 minute, 72 °c extension for 1 minute and final extension for 7 minutes. The PCR product was ran for 2 hours 45 minutes at 110 Volts in a 2.5% gel stained with Greenstar nucleic acid stain followed by visualization in BioDoc IT gel documentation.

###  1.2.4 Data Scoring

The amplified bands (alleles) were scored based on their band marker sizes for each varieties and primer combinations.

**1.2.5 Statistical Analysis**

Data were entered into a binary matrix and subsequently analyzed using the Power Marker software package [13]. The total number of alleles per locus, percentage of polymorphic alleles, low-frequency alleles (frequency of allele <30%), high-frequency alleles (frequency of allele >30%), and polymorphism information content (PIC) were calculated to assess the diversity of alleles of marker locus. Genetic similarity coefficients were calculated and used to assess the genetic relationship among the twenty cultivars, which were analyzed using the ARLEQUIN 3.01 software by analysis of molecular variance (AMOVA) [7]. Dendrogram were generated using DARwin software. Principal coordinate analysis (PCoA) and dissimilarity matrix were performed using DARwin software version 6.055. Genetic differentiation among the assumed subpopulation was analyzed using Nei’s gene diversity statistics using GenAlEx program version 6.50. The polymorphism information content (PIC) value of SSR markers was calculated using the following formula [2]:

$$PIC=1-\sum\_{i=1}^{k} Pi^{2}$$

Where k is the total number of alleles (bands) detected for one SSR locus and p is the proportion of the cultivars containing the allele (band) in all the samples analyzed.

**1. 3 RESULTS**

### 1.3.1 Simple Sequence Repeats (SSRs) Polymorphism and Population Structure of the Varieties

A total of 6 SSR primers linked to drought tolerance in the study produced scorable amplification bands that were used in the analysis (Table 2). The number of alleles per primer ranged from 3 to 5 with an average of 3.8 while the polymorphic information content (PIC) values ranged from 0.17 to 0.68 with an average value of 0.53. The primer RM390 (0.82) showed higher discriminatory power to distinguish varieties due to its high PIC value while primer RM36 (0.19) showed lower PIC value indicating less discriminatory power of this primer under study. PIC value of microsatellite marker higher than 0.5 is considered highly informative. Gene differences values of 20 upland rice varieties ranged from 0.19 (RM36) to 0.73 (RM390) with a mean of 0.58 (Table 2). These values indicated that the varieties had several different recognizable alleles related to drought tolerance. The heterozygosity ranged from 0 to 0.33 (RM3558) with an average of 0.06 across all loci. The RM3558 and RM390 loci were the only ones that detected heterozygotes in the analyzed varieties. The majority of the SSR markers exhibited heterozygosity as zero, indicating that majority of rice varieties used were clean and completely homozygous for SSR markers used in the study, which may be the result of the self-pollinated mode of reproduction of rice.

**Table 2: Number of Alleles, Polymorphism Information Content, Allele Frequency Heterozygosity and Gene Diversity Value in 20 Upland Rice varieties using 6 SSR Drought Tolerance-associated Markers**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Marker** | **Allele frequency** | **Allele number** | **PIC** | **Gene Diversity** | **Heterozygosity** |
| RM583 | 0.50 | 4 | 0.59 | 0.65 | 0 |
| RM36 | 0.90 | 3 | 0.17 | 0.19 | 0 |
| RM3558 | 0.85 | 5 | 0.63 | 0.68 | 0.33 |
| RM38 | 0.55 | 3 | 0.48 | 0.67 | 0 |
| RM390 | 0.35 | 4 | 0.82 | 0.73 | 0.02 |
| RM170 | 0.45 | 4 | 0.64 | 0.69 | 0 |
| **Mean** | **0.53** | **3.83** | **0.53** | **0.58** | **0.06** |

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##### Figure 1: Simple Sequence Repeat (SSR) Markers DNA Profile of 20 Rice varieties generated by the 6 Primers

### 1.3.2 Analysis of Molecular Variance (AMOVA)

Analysis of molecular variancewas carried out on population provided by model-based analysis, because of its determination and consistency to give detail information about the genetic constitution of the population. AMOVA revealed the presence of 12% of the variation among the populations, while, 87% of the variation among individuals and 1% of the variation among individuals within a population (Table 3). AMOVA showed that most of the variations in rice varieties mainly occurred among individuals. F statistic (FST) was 0.122, while FIS and FIT were 0.985 and 0.987. Higher FIS, which is measured at the subgroup level in the whole population, has indicated a lack of heterozygosity and high distinctness of populations, due to the autogamous nature of the crop. The determination of FST has shown high genetic variation among the population. Nei genetic distance ranged from 0.127 to 0.189. The maximum distance was observed between Pop 3 and pop 2 (0.189) and minimum distance was observed between pop1 and pop 3 (0.127), indicating that genomic differences between pop 3 and pop2 were more and it was less between pop3 and pop 2 (Table 3).

#### Table 3: Analysis of Molecular Variance (AMOVA) of 20 up Land Rice Varieties.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Source** | **df** | **SS** | **MS** | **Est. Var.** | **% of variation** |
| Among Pops | 2 | 12.275 | 6.138 | 0.232 | 12% |
| Among Individual | 17 | 56.600 | 3.329 | 1.652 | 87% |
| Within Individual | 20 | 0.500 | 0.025 | 0.025 | 1% |
| **Total** | **39** | **69.375** |  | **1.909** | **100%** |
| **F. statistics** | **Value** | **P(rand>= data** |  |  |  |
| FST | 0.122 | 0.013 |  |  |  |
| FIS | 0.985 | 0.110 |  |  |  |
| FIT | 0.987 | 0.001 |  |  |  |
| Nm | 1.807 |  |  |  |  |

#### Table 4: Pairwise Population Matrix of Nei Genetic Distance of 20 Rice Varieties at Sub-Population Levels

|  |  |  |  |
| --- | --- | --- | --- |
| **Populations** | **P1** | **P2** | **P3** |
| P1 | 0.000 |  |  |
| P2 | 0.000 | 0.000 |  |
| P3 | 0.127 | 0.189 | 0.000 |

###  1.3.3 Genetic Similarity and UPGMA Cluster Analysis

A dendrogram of the 20 upland rice varieties using UPGMA procedure clustered the varieties into two major clusters in accordance with their source locations (Figure 2). Cluster I divided into three sub-clusters which include cluster A-I, cluster A-II and Cluster A-III, while cluster II has only one group. Cluster A-I consisting of eight varieties with four groups, (Gorlu, youmo 1 and White rice), (Loriza and Youmo 2), (Jaowo and NERICA14) and Kpatawee standing alone. Cluster A-II consisting of only three group (Red Youmo, Dourado NERICA 4, and LAC-23), (MWUR, NERICA 1, 10 and11), While A-III with Komboka is standing alone. Cluster II consisting of two groups, (NERICA 2 and 3) and (L-22) (Figure 2).



##### Figure 2: A Dendrogram Generated from UPGMA Cluster Analysis Based on Jaccard’s Dissimilarity Coefficient with 1000 Boostrap Replicates Showing 20 Rice Varieties Generated from 6 SSR Markers.

Principal Coordinate Analysis (PCoA) using SSR marker allelic data determines the genetic relatedness among the upland rice cultivars. The first three principal axes explained 29.28% of the total percentage of variation and second axes explained 24.27% of the variation, while the third axes explained 12.19% of the variation (Figure 3). PCoA indicated that in population 1, NERICA 2, 3 were closely related and NERICA 14 and 11 were separated along the second coordinate (x-axis) and in population 2 Komboka, MWUR, NERICA 1,10 were grouped on second coordinate while in population 3 Red youmo, NERICA 4 and Dourado were grouped on the first coordinate. Also, in population 3 LAC -23, White rice, Loriza, youmo2, Kpatawee, Jaowo and L-22 were separated from each other. (Figure 3), this suggests that upland rice differentiation was based on the molecular data of the SSR markers used in the current study. Distinction among the upland rice cultivars was more obvious in population1and 2 than that in population 3 (Figure 3). The level of genetic diversity was similar between population 3 while population 1 and 2 showed considerable differences among the rice varieties.

##### Figure 3: Principal Coordinate Analysis of 20 Upland Rice Varieties using SSR Markers

### 1.4 DISCUSSIONS

The use of molecular markers to select rice varieties keeping genomic areas that control target traits can improve the selection for drought tolerant rice. This is because molecular markers are transferred from generation to generation and are not subject to environmental influences ([1]. The SSR markers are efficient along with the system of choice for genetic analysis in rice because of their abundance in the rice genome, high level of polymorphism, dependable and high but simple reproducible assays [22]. For these reasons therefore, SSR markers have been used in molecular characterization of rice as well as other crop species, [33].

This study indicated that there was genetic variation among 20 upland rice varieties, and hence they could be distinguished from each other. Total number of 23 alleles were produced using six Simple Sequence Repeats, of which all the alleles were found polymorphic. The polymorphic alleles play important roles in many research fields such as variety differentiation, and conservation to identify potential parents, [32] Within the 6 primers, all were reported as polymorphic markers. The number of alleles per locus detected in the present study were similar to earlier reports [10]. However, [17] observed a higher number of alleles per locus. [18] used 20 SSR markers to assess variation among 20 rice varieties, where 84 alleles were detected with an average of 2.89 alleles per locus. Similarly, [22] identified 63 alleles with an average of 2.75 alleles per locus.

The study reported 23 alleles were used as a diagnostic marker for specific varietal identification, and they could distinguish varieties from the rest of the others [11]. These alleles might play a vital role under crop stress situation so that crop can withstand drought stress conditions. In this context, these varieties could be used as potential donors for desirable traits with recurrent parents for abiotic stress tolerance in resistance breeding program. The study reported low and high frequency alleles of 0.35 and 0.90, which plays significant role in monitoring linkage between within the markers [25]. [3] reported a high frequency allele of 53.6% in the 69 varieties whereas [5] reported 85% of the population shares high-frequency allele. Three loci, namely RM3558, RM390 and RM170 amplified the highest number of high-frequency alleles in the study. Similarly, [22] reported that all the 51 primers amplify at least one high-frequency allele in many rice varieties. This reflects the high discriminatory ability of the used markers and therefore affirms their use in genetic characterization studies [22]. Moreover, according to [19] PIC value effectively demonstrates the power of SSR markers in measuring genetic variation among the varieties.

Polymorphism information content (PIC) indicates that information of a marker and alleles were wide range of genetic variation among the varieties. PIC value shows that marker is highly polymorphic, and would have an infinite number of alleles, and the marker is more informative, this suggests higher discriminatory authority of marker [6]. Simple Sequence Repeat polymorphism analysis revealed an average PIC of 0.68 for 6 markers, which reflected improved discriminatory power of these markers to reveal higher level of genetic variation among varieties and indicated the diverse nature of variety under study. Similar results for average PIC were reported by [16]. [3] reported a higher average PIC of 0.811 per locus, this might be due to the use of a more diverse set of rice varieties in their study or due to the use of highly polymorphic markers. These findings indicated that the rice varieties used in the present study material has larger genetic differences.

The AMOVA indicated that there was a higher proportion of variation among individuals and a lower proportion of variation among populations. [10] and [22] reported a huge proportion of variation among individuals in the rice population. All the varieties used in the study were collected from different regions of origin, which results in higher variation among individuals than among populations. Within individuals 1% variance was observed, it indicated the high purity of varieties and has been maintained carefully without any mixture. A very high FIT value has proven a lack of heterozygosity most likely due to the closely relatedness nature of these varieties [14]. The FST closely relatedness coefficient within subpopulations relative to the total provides a measure of the genetic differentiation between subpopulations [15]. The determination of FST using structure analysis for the subpopulation of the study was 0.407 which indicated high differentiation between subpopulation because the varieties were collected from a wide range of ecology and topography. [27] proposed that values of FST 0.25 explain a very great differentiation between subpopulations; the range of 0.15 to 0.25 indicates moderate differentiation; while differentiation is not negligible if FST is 0.05 or less.

The population structure of upland rice varieties under this study grouped them into two major clusters. The Dendrogram analysis provided an indication of the ecology of each rice varieties and the genetic relationship between them as cluster 1 and cluster 2 (with three sub-clusters). The clustering was largely depending on drought tolerance according to their band sizes based on SSR markers. The obtained results reflected the existence of considerable amount of genetic differences among the tested varieties. It also demonstrates the possibility of genetic improvement to water stress tolerance using these cultivars in breeding program. The results also demonstrated the strength of molecular analysis in assessing genetic variation. The SSR markers have been widely used to characterize rice varieties and evaluate genetic relationships among varieties. The clustering was largely depending on water stress tolerance according to their band sizes by SSR markers. The obtained results reflected the existence of considerable amount of molecular diversity among the tested varieties and hence demonstrated of the possibility of genetic improvement of water stress tolerance using those varieties in breeding program. The results also demonstrated the strength of molecular analysis in assessing genetic variation. The SSR markers have been widely used to characterize rice varieties and evaluate genetic relationships among varieties. A Dendrogram was constructed based on the similarity index. Clustering represented the genetic similarity among the varieties as well as their habitat adaptation. Therefore, it could be assumed from the results that the markers used in this study were able to group the varieties based on their degree of water stress tolerance, reflecting the power of the SSR markers in analyzing and explaining the population genetic structure as earlier demonstrated by, [31] Also, it was observed that the NERICA varieties showed better performance under the current study conditions.

The genetic distances of 20 upland rice varieties were analyzed using principal coordinate analysis (PCoA). Population one was separated from population two, but the genetic distance between the two was not far, and the relationship between these upland rice varieties was relatively closed or homologous. These results are in agreement with [5] who reported the ability of PCoA technique to partition rice varieties due to the variation in molecular and morphological data. Principal coordinate Analysis (PCoA) provided an insight of the contribution of each of the trait towards divergence among the characteristics of upland rice varieties. The PCoA analysis revealed that maximum differences in a population of 20 rice varieties was directed by traits. Principal coordinate analysis grouped the rice varieties into different clusters indicating the presence of considerable phenotypic diversity among the varieties. The result highlights the source of the rice varieties as a major factor influencing their genetic makeup, with Pop3 having a higher number of varieties from specific sources (local landraces Liberia respectively). However, among Pop 2, NERICA 1 and 10 were closely related while in Pop1, NERICA 2 and 3 were also closely related indicating that these varieties could have some genetic relativeness and have the gene of water stress tolerance. Similarities among 20 diverse rice varieties from different sources were also reported by, [30] Principal Coordinates analysis (PCoA) was used to confirm the patterns of admixture among populations. The analysis of rice varieties using the PCoA revealed that the NERICA rice varieties including MWUR were grouped into clusters.

**1.5 CONCLUSION**

The study concluded that among the markers, RM3558, RM390 and RM170 were markers linked to drought tolerance. These markers have the potential to differentiate tolerant varieties from susceptible ones. Based on the SSRs difference analysis and clustering patterns, the NERICA rice varieties were identified as diverse cultivars and they are suitable and adapted to areas with insufficient rain fall for upland rice production for future breeding program.

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